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THE UNIVERSITY OF SYDNEY

PROVISIONAL SPECIFICATION

Invention Title:

Intracellular feedback controls in the diagnosis and treatment of human disease

The invention is described in the following statement:

Field of the Invention:

The present invention relates to the diagnosis and treatment of human disease, particularly human disease characterised by abnormal cytosolic ion composition resulting from reduced or otherwise over activity of Na^+ transport proteins such as the ubiquitous Na^+-H^+ exchanger, NHE1.

Background of the Invention:

In recent years, over activity of Na^+ transporting systems in absorptive epithelia has been implicated in the pathogenesis of a number of major diseases including hypertension (1, 2), diabetic nephropathy (3), cardiological syndrome X (4), ventricular hypertrophy (5), chronic pulmonary hypertension (6) and cystic fibrosis (7). In the case of the hereditary hypertensive disease known as Liddle's syndrome, this activity has been attributed to a mutation of the epithelial Na^+ channel leading to loss of an inhibitory feedback mechanism which normally switches off Na^+ channel activity in response to increased intracellular Na^+ (8, 9). The mechanisms that underlie this so-called homocellular regulation have been the subject of controversy, but recent experiments have revealed a previously unsuspected mechanism in which cytosolic Na^+ is "sensed" by an intracellular receptor (10). This receptor activates the G protein, G_o (11), the α-subunit of which then causes the ubiquitin-protein ligase, Nedd4 (10), to ubiquitinate and inactivate the epithelial Na^+ channels (12, 13). This receptor for intracellular Na^+ is blocked by amiloride and amiloride analogs such as dimethylamiloride and benzimidazole guanidinium (10), thus explaining the previously puzzling ability of these agents to stimulate Na^+ channel activity (14).

The present applicants have now found that the intracellular Na^+ receptor that controls absorptive epithelial Na^+ channels also controls the activity of the ubiquitous isoform of the Na^+-H^+ exchanger, NHE1. This finding suggests that intracellular Na^+ receptors form part of a general mechanism for regulating Na^+ transport proteins. It is therefore anticipated that the intracellular Na^+ receptors (and the signal-transduction systems by which they control Na^+ channels, Na^+-H^+ exchangers and other Na^+ transporting proteins) shall provide a useful target for diagnostic assays and treatments for hypertension and other diseases.

Disclosure of the Invention:

Thus, in a first aspect, the present invention provides a method of treatment of a human disease which is characterised by abnormal cytosolic ion composition resulting from reduced or otherwise over activity of a Na^+ transport protein, the method comprising administering to a subject having said disease an effective amount of an agent that substantially restores the ion composition of the cytosol in diseased cells to that which is found in corresponding cells from healthy tissue.

Preferably, the present invention provides a method of treatment of a human disease which is characterised by abnormal cytosolic ion composition resulting from reduced or otherwise over activity of a Na^+ transport protein other than an epithelial Na^+ receptor. Most preferably, the present invention provides a method of treatment of a human disease which is characterised by abnormal cytosolic ion composition resulting from reduced or otherwise over activity of a Na^+ transport protein selected from the group consisting of NHE1, NHE3 and the Na^+K^+2Cl cotransport protein.

Where the characteristic abnormal cytosolic ion composition arises from reduced Na^+ transport protein activity resulting from, for example, Na^+ transport protein mutation (e.g. hereditary), depressed Na^+ transport protein expression or inappropriate activity of the Na^+ transport protein inhibitory feedback mechanism, the administered agent may be selected from gene therapy agents (e.g adenoviruses capable of causing the expression of non-mutated Na^+ transport protein) and agents capable of blocking the Na^+ transport protein inhibitory feedback mechanism. Preferred agents of the latter kind are amiloride and amiloride analogs (e.g. 6-iodoamiloride, N-dimethylamiloride, and benzimidazoylguanidium), G-protein inhibitors (e.g. GDP-β-S and NF023) and agents that inhibit the action of ubiquitin protein ligase on the Na^+ transport protein. Examples of this latter kind of agents are dominant negative mutants of ubiquitin (e.g. K48R), agents that prevent binding of the ubiquitin protein ligase to the Na^+ transport protein (e.g. membrane permeable peptide analogs of the protein motif to which the ubiquitin protein ligase binds such as the WW2 and WW3 domains of Nedd4), agents that prevent ubiquitination of the Na^+ transport protein (e.g. membrane permeable peptide analogs of the protein motif which is actually ubiquitinated (e.g. the N-terminal of the β-subunit of ENaC) and inhibitors of the effectors of ubiquitin action on the Na^+ transport protein including

proteins involved in endocytosis (e.g. membrane permeable analogs of amphiphysin SH3 peptide) and degradation of the Na^+ transport protein by proteasomes (e.g. lactacystin) or lysosomes (e.g. bafilomycin or chloroquine). Peptide analogs may be made to be membrane permeant by including a *Drosophila* antennapedia homeobox domain (15, 16).

Where the characteristic abnormal cytosolic ion composition arises from Na^+ transport protein over activity resulting from, for example, Na^+ transport protein mutation (e.g. hereditary), loss of the Na^+ transport protein inhibitory feedback mechanism or inappropriate activity of other control systems (e.g. excessive levels of growth factors or glucose), the administered agent may be selected from gene therapy agents (e.g. adenoviruses capable of causing the expression of a protein participating in the Na^+ transport protein inhibitory feedback mechanism), intracellular Na^+ receptor activators (e.g. guanidium and guanidium analogs), G-protein activators (e.g. GTP-γ-S and receptor mimetic peptides such as APP20(17)), ubiquitin ligase activators (e.g. membrane permeable peptides that mimic the effect of active G proteins on the ubiquitin protein ligase), and agents that trigger endocytosis.

In a second aspect, the present invention provides a method of diagnosis of a human disease which is characterised by abnormal cytosolic ion composition resulting from reduced or otherwise over activity of a Na^+ transport protein, the method comprising isolating from a subject suspected of having said disease a sample of diseased cells (such as epithelial cells or lymphocytes) and assessing said sample for reduced or otherwise over activity of said Na^+ transport protein or its inhibitory feedback mechanism.

Preferably, the present invention provides a method of diagnosis of a human disease which is characterised by abnormal cytosolic ion composition resulting from reduced or otherwise over activity of a Na^+ transport protein other than an epithelial Na^+ receptor. Most preferably, the present invention provides a method of diagnosis of a human disease which is characterised by abnormal cytosolic ion composition resulting from reduced or otherwise over activity of a Na^+ transport protein selected from the group consisting of NHE1, NHE3 and the Na^+K^+2Cl cotransport protein.

The sample of diseased cells may be assessed for reduced or over activity of Na^+ transport protein by, for example, determining the rate of Na^+-dependent intracellular pH (pH_i) recovery and comparing the value against similarly measured values from cells from healthy tissue isolated

from the said suffering subject or a panel of control (i.e. non-diseased) subjects.

In a variation of the invention according to the second aspect, the sample of diseased cells may be assessed for over or under expression of the Na^+ transport protein or another protein participating in the Na^+ transport protein inhibitory feedback mechanism (e.g. by polymerase chain (PCR) techniques, Northern blot hybridisation, Western blot or immunoprecipitation).

In a third aspect, the present invention provides a method of diagnosis of a human disease which is characterised by abnormal cytosolic ion composition resulting from reduced or otherwise over activity of a Na^+ transport protein, the method comprising isolating a genomic DNA sample from a subject suspected of having said disease and assessing said sample for the presence of a gene encoding a mutated product causitive of said reduced or over activity of said Na^+ transport protein.

In a fourth aspect, the present invention provides a method of assessing a subject for a predisposition to a human disease which is characterised by abnormal cytosolic ion composition resulting from reduced or otherwise over activity of a Na^+ transport protein, the method comprising isolating a genomic DNA sample from a subject and assessing said sample for the presence of a gene encoding a mutated product causitive of reduced or over activity of said Na^+ transport protein.

In the methods of the third and fourth aspects, the human disease is preferably one which is characterised by abnormal cytosolic ion composition resulting from reduced or otherwise over activity of a Na^+ transport protein other than an epithelial Na^+ channel (e.g. NHE1, NHE3 and the Na^+K^+2Cl cotransport protein). The genomic DNA sample may be isolated using routine protocols known to the art. The genomic DNA sample may be isolated from any cell sample such as whole blood, tissue biopsy or cheek cell sample. The assessment of the presence of a gene encoding a mutated product causitive of reduced or over activity of the Na^+ transport protein, may be preferably achieved by hybridisation or PCR techniques using probes/primers designed to specifically hybridise to genes including mutated nucleotide sequences. The gene whose presence is to be assessed may encode a mutated Na^+ transport protein or a mutated protein participating in

the Na^+ transport protein inhibitory feedback mechanism (e.g. a mutated G-protein or mutated intracellular Na^+ receptor).

The methods of the invention are applicable to, for example, hypertension, renal failure, cardiac hypertrophy and cardiological syndrome X.

The present applicants have also found that the intracellular Na^+ receptor controlling NHE1 is blocked by amiloride and amiloride analogs with the following order of potency:

6-iodoamiloride (EC_{50} = 0.1 μmol/l) < amiloride (1.0 μmol/l) < 5-N-dimethylamiloride (30 μmol/l), benzamil (> 30 μmol/l) < benzimidazolylguanidium (300 μmol/l)

Knowledge of these differing potencies enables the isolation of a DNA molecule encoding the intracellular Na^+ receptor controlling NHE1. That is, by using the α-subunit of G_o as "bait" in a yeast two-hybrid technique ("The yeast two-hybrid system" edited by P.L. Bartel & S. Fields, Oxford University Press, Oxford, 1997), DNA molecules encoding interacting proteins may be isolated from suitable cDNA or genomic DNA libraries and then screened for the ability of the encoded proteins to bind 6-iodoamiloride. Further screens may be conducted for the relative inability of the encoded proteins to bind benzamil, the ability of antibodies raised to the encoded proteins to immunoprecipitate the α-subunit of G_o, and the ability of antibodies raised to the encoded proteins to block the NHE1 inhibitory feedback mechanism.

By using the yeast two-hybrid system with a constitutively active mutant of the α-subunit of G_o, it is possible to identify and isolate proteins which interact with active G_o and hence are involved in the inhibitory feedback mechanism at a loci downstream of G_o. Similarly, by using the yeast two-hybrid system with a dominant negative mutant of the α-subunit of G_o, it is possible to identify and isolate proteins such as the intracellular Na^+ receptors which are involved in the inhibitory feedback mechanism at a loci upstream of G_o.

The present applicants have isolated 5 cDNA molecules from mouse kidney and mandibular gland cDNA libraries encoding candidates for the intracellular Na^+ receptor controlling NHE1 and Na^+ channels. The 5 candidates are nucleobindin (18), GAIP (19), rap1GAP (20) and novel proteins designated c10a and SCunique.

Thus, in a fifth aspect, the present invention provides an isolated DNA molecule encoding a candidate intracellular Na^+ receptor designated C10a, said DNA molecule comprising a nucleotide sequence substantially corresponding to that shown as SEQ ID NO: 1 or a nucleotide sequence showing ≥ 75% (more preferably ≥ 85%, most preferably ≥ 95%) homology to that shown as SEQ ID NO: 1.

Preferably, the isolated DNA of the fifth aspect encodes a protein comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 2.

In a sixth aspect, the present invention provides an isolated DNA molecule encoding a candidate intracellular Na^+ receptor designated SCunique, said DNA molecule comprising a nucleotide sequence substantially corresponding to that shown as SEQ ID NO: 3 or a nucleotide sequence showing ≥ 75% (more preferably ≥ 85%, most preferably ≥ 95%) homology to that shown as SEQ ID NO: 3.

Preferably, the isolated DNA of the sixth aspect encodes a protein comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 4.

The isolated DNA molecule of the fifth and sixth aspect may be incorporated into plasmids or expression vectors (including viral vectors), which may then be introduced into suitable bacterial, yeast, insect and mammalian host cells. Such host cells may be used to express the receptor encoded by the isolated DNA molecule.

Accordingly, in a seventh aspect, the present invention provides a mammalian, insect, yeast or bacterial host cell transformed with the DNA molecule of the fifth or sixth aspect.

In an eighth aspect, the present invention provides a method of producing an intracellular Na^+ receptor, comprising culturing the host cell of the seventh aspect under conditions enabling the expression of the DNA molecule and optionally recovering the expressed receptors.

Preferably, the host cell is mammalian, amphibian or of insect origin. Where the cell is mammalian, it is presently preferred that it be a Chinese hamster ovary (CHO) cell or human embryonic kidney 293 cell. Where the cell is of amphibian origin, it is presently preferred that it be a Xenopus oocyte. Finally, where the cell is of insect origin, it is presently preferred that it be an insect Sf9 cell.

In a ninth aspect, the present invention provides a candidate intracellular Na^+ receptor designated C10a, said receptor comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 2, in a substantially pure form.

In a tenth aspect, the present invention provides a candidate intracellular Na^+ receptor designated Scunique, said receptor comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 4, in a substantially pure form.

In an eleventh aspect, the present invention provides an antibody capable of specifically binding to a receptor according to the ninth or tenth aspect.

In a twelfth aspect, the present invention provides a method for detecting agonist or antagonist agents of the receptor of the ninth or tenth aspect, comprising contacting said receptor, or a cell transfected with and expressing the DNA molecule of the fifth or sixth aspect, with a test agent under conditions enabling the activation of said receptor, and detecting an increase or decrease in activity of the receptor.

In a further aspect, the present invention provides a nucleic acid probe/primer comprising a nucleotide sequence of 10 or more nucleotides capable of specifically hybridising to a unique sequence within the DNA molecule of the fifth or sixth aspect.

The term "substantially corresponding" as used herein in relation to nucleotide sequences is intended to encompass minor variations in the nucleotide sequence which due to degeneracy in the DNA code do not result in a change in the encoded protein. Further, this term is intended to encompass other minor variations in the sequence which may be required to enhance expression in a particular system but in which the variations do not result in a decrease in biological activity of the encoded protein.

The term "substantially corresponding" as used herein in relation to amino acid sequences is intended to encompass minor variations in the amino acid sequences which do not result in a decrease in biological activity of the encoded protein. These variations may include conservative amino acid substitutions. The substitutions envisaged are:-

G, A, V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, Nα-alkalamino acids.

References to percent homology values herein are calculated by the BLAST program blastn as described by Altschul, S.F. et al., "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Research Vol. 25, No. 17, pp 2289-3402 (1997).

The terms "comprise", "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated step, component or feature or group of steps, components or features with or without the inclusion of a further step, component or feature or group of steps, components or features.

The invention will hereinafter be further described by way of the following non-limiting example and accompanying figures.

Brief description of the accompanying figures:

Figure 1: Shows features of the Na^+-dependent pH_i recovery measured with a zero Na^+ pipette solution. (A) Representative experiment with 10 mM ATP in the pipette. The bar indicates the period of readmission of 155 mM Na^+ solution to the bath. (B) Concentration-response relation for the effect of extracellular ethylisopropylamiloride (EIPA) on the Na^+-dependent pH_i recovery. (C) The effect of modifying intracellular ATP levels.

Figure 2: Shows inhibition of Na^+ -dependent pH_i recovery by cytosolic Na^+. (A) Dependency of the Na^+-dependent pH_i recovery on pipette Na^+. (B) The effects of inclusion of 20 mM $NMDG^+$ in the zero Na^+ pipette solution, or by buffering intracellular and extracellular Ca^{2+} to zero by the inclusion of 20 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA) in the pipette solution and 1 mM EGTA in the bath solution. No Ca^{2+} was added to either solution.

Figure 3: Shows that the Na^+ feedback inhibition is mediated by a G protein. (A) The effect of the addition of 100 μM GDP-β-S to the pipette solution. (B) The effect of the addition of 500 ng/ml activated pertussis toxin to the pipette solution. (C) The effect of the addition to the pipette solution of antibodies directed against various G protein α-subunits [$AbG_{i1,i2}$ = against C terminals of $G\alpha_{i1}$ and $G\alpha_{i2}$; $AbG_{o.i3}$ = against C terminals of $G\alpha_o$ and $G\alpha_{i3}$; AbG_{i3} = against C terminal of $G\alpha_{i3}$; AbG_o = against N terminal of $G\alpha_o$; all 1 in 200 (vol/vol)].

Figure 4: Shows the inhibition of Na^+ feedback by intracellular amiloride. (A) Concentration-dependency of the effect of intracellular amiloride when included in 20 mM Na^+ solution. (B) The effect of the inclusion of 0.2 μM activated recombinant α-subunit of G_o (act G_o) and amiloride (10 and 30 μM) in the zero Na^+ pipette solution. AS and inact G_o denote controls in which activation solution or inactive $G\alpha_o$, respectively, were added to the pipette solution. (C) The effect of the inclusion of anti-Nedd4 antibody (A-Nd4; 1 μg purified 1gG/ml). GST-WW fusion protein (G-W; 0.3 mg/ml). GST-wild type-ubiquitin (wt; 0.3 mg/ml) or GST-dominant negative-ubiquitin (K48R) fusion protein (dn; 0.3 mg/ml) in the 20 mM Na^+ pipette solution. In A and C the broken lines indicate the mean rate of pH_i recovery observed with zero Na^+ pipette solution.

Figure 5: Shows the mechanisms of feedback inhibition by intracellular Na^+ of epithelial Na^+ channels in salivary duct (absorptive) cells (A) and Na^+-H^+ exchange in salivary endpiece (secretory) cells (B). In each cell model, the apical membrane is on the left and the sodium pump (Na^+, K^+ ATPase) is shown in the basolateral membrane on the right.

Example:

Materials and Methods.

Cell Preparation. Male Quackenbush strain mice were killed by cervical dislocation, and the mandibular glands were removed, finely minced, and incubated for 12 min in a physiological salt solution containing 1 mg/ml collagenase (Worthington type IV). The cell suspension was then dispersed by trituration and washed with fresh Na^+ rich bath solution containing 145 mM NaCl, 5.5 mM KCl, 1.2 mM $MgCl_2$, 7.5 mM Na-Hepes, 7.5 mM H-Hepes, 1 mM $CaCl_2$ and 10 mM glucose; the pH was adjusted to 7.4 with NaOH. The cells were filtered through a 75-μm nylon mesh and kept on ice until required.

Patch-Clamp Techniques. A technique based on that of Demaurex and coworkers (21) was used in which the whole-cell patch-clamp technique is used to control cytosolic composition while the pH-sensitive dye, BCECF, is used to measure intracellular pH (pH_i). The patch-clamp techniques used were are described (22), and the cells were loaded with BCECF by including it in the pipette solution. Except for the experiments summarised in Figure 1C, in which $MgSO_4$ replaced MgATP, pipettes were filled with solutions

containing 145 mM K-glutamate and Na-glutamate combined, 5 mM KCl, 5 mM Mes, 10 mM Mg-ATP, 1 mM EGTA, 40 mM sucrose, and 0.2 mM BCECF; the pH was adjusted to 6.0.

Measurement of pH_i. The equipment used to measured pH_i was as described (23). The chamber (0.3 ml) was continuously perfused with a Na^+-free bath solution containing 145 mM *N*-methyl-D-glucamine (NMDG)-Cl, 5.5 mM KCl, 15 mM H-Hepes, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose with a pH of 7.4. Single cells in the whole-cell configuration were voltage-clamped at -30 mV. After 3 min they were illuminated alternately at 490 and 430 nm. Na^+-H^+ exchange activity was measured by reintroducing Na^+ to the bath between 2 and 3 min after the start of illumination. pH_i recovery rate was determined by fitting a linear regression to the linear phase of the pH_i recovery (i.e., between 20% and 80% of maximal recovery). Calibration of the BCECF signal was by the nigericin high-K^+ method (23).

Chemicals. Sources of chemicals and the methods for activating pertussis toxin and G protein α-subunits were as reported (24, 25). Antibodies directed against the C terminals of the α-subunits of G_{i1}/G_{i2}, G_{i3} and G_{i3}/G_o were obtained from Calbiochem, and antibodies against the N-terminal of the α-subunit of G_o were obtained from DuPont-NEN. They were used in the pipette solution at a 1 in 200 (vol/vol) dilution of the solution provided by the manufacturer. Glutathione-S-transferase (GST)-WW (G-W), GST-dominant negative-ubiquitin (K48R), and GST-wild type-ubiquitin fusion proteins were produced as described (24). The anti-Nedd4 antibody (A-Nd4) was purified IgG raised in rabbits against the C-terminal half of the protein (24, 26).

Results are presented as means ± SEM. At least five cells were tested in each experimental group. Statistical significance was assessed by using Student's unpaired t test. All experiments were performed at 22°C.

Results.

Activity of Na^+-H^+ exchangers was measured by a technique described by Demaurex and coworkers (21) in which the whole-cell configuration of the patch-clamp technique is used to control cytosolic composition while the pH-sensitive dye, BCECF, measures pH_i. The cells were bathed initially in a zero Na^+ solution so that they would be unable to oppose the acid load imposed by the pipette solution using Na^+-dependent H^+ transporters such as the Na^+-H^+ exchanger. The bath solution then was changed to one containing 155

mM Na^+ so as to activate the Na^+-H^+ exchanger and cause pH_i to recover toward normal levels (Fig 1A). The rate of this Na^+-dependent pH_i recovery was used to estimate Na^+-H^+ exchange activity. The technique was validated by demonstrating that Na^+-dependent pH_i recovery has features consistent with its being the result of the NHE1 isoform of Na^+-H^+ exchanger, which predominates in salivary secretory cells. It was found that the Na^+-dependent pH_i recovery was highly sensitive to the amiloride analog, ethylisopropylamiloride (Fig. 1B), and that the recovery depended on the presence of ATP (21), being inactivated when intracellular ATP was depleted by treatment with 2-deoxy-D-glucose (5 mM) and oligomycin (5 μg/ml; Fig. 1C).

It was demonstrated that the rate of the Na^+-dependent pH_i recovery declined with increasing pipette Na^+ concentration (Fig. 2A) in a manner similar to that described in sheep F2 Purkinje fibres (27). This inhibition evidently was caused by increased $[Na^+]_i$, because it could not be reproduced by the large organic cation, $NMDG^+$ (Fig. 2B). Because intracellular free Ca^{2+} is known to regulate Na^+-H^+ exchangers (28), an investigation was made to determine whether a change in free intracellular Ca^{2+} concentration could mediate this phenomenon. It was found that buffering cytosolic and extracellular Ca^{2+} to nominal zero did not alter the effect of increased $[Na^+]_i$ (Fig. 2B).

An investigation was also made to determine the mechanism by which $[Na^+]_i$ controls the activity of the Na^+-H^+ exchanger. It was found that inclusion of the pipette solution of 100 μM GDP-β-S (which competitively inhibits the binding of GTP by G proteins; ref. (29) or of 500 ng/ml activated pertussis toxin (which ADP ribosylates G proteins of the G_i and G_o classes so as to prevent their interaction with receptors; ref. (30), reversed the inhibitory effect of 20 mM Na^+ (Fig. 3A and B). The ability of these agents to overcome the inhibitory effect of raised intracellular Na^+ completely without altering the electrochemical gradient for Na^+ indicates that the inhibition is not caused by a decreased electrochemical driving force for Na^+-H^+ exchange. Rather, it must be caused by a G protein-mediated feedback pathway. In this regard, it was further found that inclusion in the pipette solution of antibodies directed against the α-subunit of the G_o protein, which is known to be expressed in salivary endpiece cells (31), abolished the

inhibitory effect of 20 mM Na^+. In contrast, antibodies directed against the α-subunits of G_{i1}, G_{i2}, and G_{i3} were without effect (Fig. 3C).

In the absorptive cell of the salivary duct, $[Na^+]_i$ is sensed by a receptor the effect of which is mediated by G_o (10). This receptor is blocked by amiloride and amiloride analogs such as dimethylamiloride and benzimidazolylguanidinium, thus explaining the ability of these agents to stimulate Na^+ channel activity. It was found that the inclusion of amiloride in the pipette solution reversed the inhibitory effect of 20 mM Na^+ (Fig. 4A). Further, it was found that the inclusion of the activated α-subunit of G_o in the zero Na^+ pipette solution (Fig. 4B) inhibited the Na^+-H^+ exchanger and that the inclusion of as much as 30 μM amiloride in the pipette solution was unable to overcome this inhibition (Fig. 4B). Thus, amiloride exerts its inhibitory action upstream of G_o, presumably at the putative receptor for intracellular Na^+.

Discussion.

It has been previously shown that $[Na^+]_i$ and the G protein, G_o, regulate the activity of the epithelial Na^+ channel in the duct cells of the mouse mandibular gland via Nedd4 (24), a ubiquitin-protein ligase that is believed to bind to Na^+ channels and regulate their activity by ubiquitinating them (12, 13). Here, it was found that feedback inhibition of the Na^+-H^+ exchanger was not prevented by inclusion in the pipette solution of an antibody directed against Nedd4 or of a fusion protein composed of GST and the three WW-domains of mouse Nedd4 (GST-W), which acts as a dominant negative mutant of Nedd4 (Fig. 4C). This finding is consistent with the low level of expression of Nedd4 in endpiece cells (24). Feedback inhibition was blocked, however, by inclusion of a dominant negative mutant of ubiquitin (K48R) (24) in the pipette solution (Fig. 4C), indicating that feedback regulation of the exchanger nevertheless is mediated by ubiquitination. Because our preliminary data show that NHE1 transfected into COS cells is ubiquitinated (data not shown), the findings indicate that feedback regulation of NHE1 is mediated by ubiquitination of the exchanger protein. The control system then would resemble the control of surface expression of epithelial Na^+ channels by ubiquitination of the channel protein catalysed by Nedd4. It cannot be excluded however, that the inactivation of NHE1 produced by Na^+ feedback is the result of ubiquitination of a protein associated with the exchanger, as recently has been proposed for the control of the growth

hormone receptor by ubiquitination (32). Whatever the mechanism, the present findings taken together with the finding that activity of epithelial Na^+ channels can be rapidly down-regulated by ubiquitination suggest that ubiquitination may be a general mechanism for the rapid control of membrane transport protein activity.

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Sequence Listing:

Applicant: The University of Sydney

Title of Invention: Intracellular feedback controls in the diagnosis and treatment of human disease

Number of SEQ ID NOs: 4

SEQ ID NO: 1

Length: 3150

Type: DNA

Organism: Mus musculus

Sequence: 1

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tacttctacc tgaaggagta tgcccgggcc ctgcagttct acaaacatga cttgctgctg 180
gcacggacca ttggtgaccg catgggggag gccaaggcta gtgggaacct gggcaacaca 240
ctcagggccc taggccgatt cgatgaggca atcgtctgct gccaacgaca cttggacatt 300
gcccaggagc aggggggacaa ggttggggag gcgagagcac tctacaacat tggaaatgtg 360
taccacgcca agggcaaaca gctttcctgg aatgctgcac aggaccccgg gcacctgcca 420
cctgatgtcc gcgagacact gcacagggcc tttgagtttt atgggaggaa cctgtctttg 480
gtgaaggaac taggcgaccg ggcggcccag ggcagggcct atggcaacct gggtaacacc 540
cactacctac tgggaaactt cacggaggcc acaaccttcc acaaagagcg cctggccatc 600
gccaaggagt ttggggacaa ggcagctgag cggagggcct acagcaacct gggcaatgct 660
cacatcttct tggggcgctt tgatgtggct gctgaacatt acaagaagac gctgcagctg 720
tctcggcagc tccgggacca ggcagtggag gctcaggctt gctacagcct gggcaacacc 780
tacacactgc tacaggacta cgagcgtgct gctgagtacc acctgcggca cctagtcatt 840
gcccaggagc tggctgatag ggtgggagag ggccgagcat gctggagcct ggggaacgcc 900
tatgtgtcca tggggagccc tgcacaggcc ttgacctttg ccaagaaaca tctgcagatc 960
tcccaggaga ttggagaccg aaacggagaa ctgacagccc gcatgaatat tgcccacctg 1020
cagctggccc tgggccggct gactagccca gcagcagcgg agaagccaga tctggctggc 1080
tatgaggcac aaggagcaag acccaaacgg acacagaggc tgagtgccga gacctgggac 1140
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ccagatgcca ttgagaggag accccgggag ggtagtcact ctccactgga cagtgctgat 1320
gtaagggtac aggtgcctcg tacgggtatt cccagggccc cgtcttctga cgaggagtgt 1380
ttctttgacc tgctgagtaa gttccagagc agtcgcatgg atgaccagcg ctgtcccctg 1440
gaggaaggcc aggctggggc tgctgaggcc acagctgccc catccgtgga ggatagagca 1500
gctcagtcct ccgtgacagc ttcaccacag acagaggagt tctttgacct cattgccagc 1560
tcccagagcc gccggctgga cgaccagagg gctagcgtag gcagcctgcc tgggctacgc 1620
atcaccctca acaatgtggg gcacctccga ggcgacgggg acgcccagga gccgggggat 1680
gagtttttca acatgcttat caaataccag tcctccagga ttgatgacca gcgctgccca 1740
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cagagggtgc aggctaagcg gatggatgag cagcgtgtgg accttgctgg gagtccagag 1860
caagaggcca gtgggctgcc tgatccccag cagcagtgtc caccgggtgc cagctaaggc 1920
ctcgccccta cagccagcca taccctactc tggactctgt aggctcacgg ttgtccacag 1980
tggccatgat cccccaataa gccaaatctt cccaaggcca tgatggacag ccagctcagc 2040
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gggtgcaggc tagagctgct atatggggga gggcatgctt ctgtccccat ggatctccac 2160
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cctagttctt gcctacaagt cctataggta gccctactgg agggctctgt acaactgtct 2880
aacccagggc ctcttaccca gagggccagg gcaagggaaa ctgaggaccc aacttctgag 2940
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ggacatggtg gccgccccgc atacttgaat gtacatgcgt atttattgct tacatgtgtt 3060
tgccatgttg ttcatgggtc ctttctgacc cgagaggtac atttgttttg ttttacccaa 3120
aaataaaagt ctgccaagtg aaaaaaaaaa 3150

SEQ ID NO: 2

Length: 638

Type: PRT

Organism: Mus musculus

Sequence: 2

Phe Phe Cys Ala Arg Arg Gly Thr Ser Arg Gln Gly Val Ala Phe Phe
1 5 10 15

Glu Ala Val Val Gln Val Gly Ile Lys Asp Leu Lys Thr Leu Ser Ala
20 25 30

Ile Tyr Ser Gln Leu Gly Asn Ala Tyr Phe Tyr Leu Lys Glu Tyr Ala
35 40 45

Arg Ala Leu Gln Phe Tyr Lys His Asp Leu Leu Leu Ala Arg Thr Ile
50 55 60

Gly Asp Arg Met Gly Glu Ala Lys Ala Ser Gly Asn Leu Gly Asn Thr
65 70 75 80

Leu Arg Ala Leu Gly Arg Phe Asp Glu Ala Ile Val Cys Cys Gln Arg
85 90 95

His Leu Asp Ile Ala Gln Glu Gln Gly Asp Lys Val Gly Glu Ala Arg
100 105 110

Ala Leu Tyr Asn Ile Gly Asn Val Tyr His Ala Lys Gly Lys Gln Leu
115 120 125

Ser Trp Asn Ala Ala Gln Asp Pro Gly His Leu Pro Pro Asp Val Arg
130 135 140

Glu Thr Leu His Arg Ala Phe Glu Phe Tyr Gly Arg Asn Leu Ser Leu
145 150 155 160

Val Lys Glu Leu Gly Asp Arg Ala Ala Gln Gly Arg Ala Tyr Gly Asn
165 170 175

Leu Gly Asn Thr His Tyr Leu Leu Gly Asn Phe Thr Glu Ala Thr Thr
180 185 190

Phe His Lys Glu Arg Leu Ala Ile Ala Lys Glu Phe Gly Asp Lys Ala
195 200 205

Ala Glu Arg Arg Ala Tyr Ser Asn Leu Gly Asn Ala His Ile Phe Leu
210 215 220

Gly Arg Phe Asp Val Ala Ala Glu His Tyr Lys Lys Thr Leu Gln Leu
225 230 235 240

Ser Arg Gln Leu Arg Asp Gln Ala Val Glu Ala Gln Ala Cys Tyr Ser
245 250 255

Leu Gly Asn Thr Tyr Thr Leu Leu Gln Asp Tyr Glu Arg Ala Ala Glu
260 265 270

Tyr His Leu Arg His Leu Val Ile Ala Gln Glu Leu Ala Asp Arg Val
275 280 285

Gly Glu Gly Arg Ala Cys Trp Ser Leu Gly Asn Ala Tyr Val Ser Met
290 295 300

Gly Ser Pro Ala Gln Ala Leu Thr Phe Ala Lys Lys His Leu Gln Ile
305 310 315 320

Ser Gln Glu Ile Gly Asp Arg Asn Gly Glu Leu Thr Ala Arg Met Asn
325 330 335

Ile Ala His Leu Gln Leu Ala Leu Gly Arg Leu Thr Ser Pro Ala Ala
340 345 350

Ala Glu Lys Pro Asp Leu Ala Gly Tyr Glu Ala Gln Gly Ala Arg Pro
        355   360   365

Lys Arg Thr Gln Arg Leu Ser Ala Glu Thr Trp Asp Leu Leu Arg Leu
    370   375   380

Pro Leu Asp Arg Glu Gln Asn Gly Glu Thr His His Thr Gly Asp Trp
385   390   395   400

Arg Gly Pro Gly Arg Asp Ser Leu Pro Leu Pro Met Arg Ser Arg Lys
                405   410   415

Tyr Gln Glu Gly Pro Asp Ala Ile Glu Arg Arg Pro Arg Glu Gly Ser
            420   425   430

His Ser Pro Leu Asp Ser Ala Asp Val Arg Val Gln Val Pro Arg Thr
        435   440   445

Gly Ile Pro Arg Ala Pro Ser Ser Asp Glu Glu Cys Phe Phe Asp Leu
    450   455   460

Leu Ser Lys Phe Gln Ser Ser Arg Met Asp Asp Gln Arg Cys Pro Leu
465   470   475   480

Glu Glu Gly Gln Ala Gly Ala Ala Glu Ala Thr Ala Ala Pro Ser Val
                485   490   495

Glu Asp Arg Ala Ala Gln Ser Ser Val Thr Ala Ser Pro Gln Thr Glu
            500   505   510

Glu Phe Phe Asp Leu Ile Ala Ser Ser Gln Ser Arg Arg Leu Asp Asp
        515   520   525

Gln Arg Ala Ser Val Gly Ser Leu Pro Gly Leu Arg Ile Thr Leu Asn
    530   535   540

Asn Val Gly His Leu Arg Gly Asp Gly Asp Ala Gln Glu Pro Gly Asp
545 550 555 560

Glu Phe Phe Asn Met Leu Ile Lys Tyr Gln Ser Ser Arg Ile Asp Asp
565 570 575

Gln Arg Cys Pro Pro Pro Asp Val Leu Pro Arg Gly Pro Thr Met Pro
580 585 590

Asp Glu Asp Phe Phe Ser Leu Ile Gln Arg Val Gln Ala Lys Arg Met
595 600 605

Asp Glu Gln Arg Val Asp Leu Ala Gly Ser Pro Glu Gln Glu Ala Ser
610 615 620

Gly Leu Pro Asp Pro Gln Gln Gln Cys Pro Pro Gly Ala Ser
625 630 635

SEQ ID NO: 3

Length: 2372

Type: DNA

Organism: Mus musculus

Sequence: 3

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cgcaaagcta ctggtctccg tcctggagca gggcttgtca ccaaagcacc gtgtcacctg 240
gctgcagact atccgaatcc tatcccgaga ccgcagctgc ctggactcat ttgccagccg 300
ccagagctta catgcactag cctgctatgc tgacattacc gtctcagagg aacccatccc 360
acagtcccca gacatggatg tcctcctcga gtctctcaaa tgcctgtgta atcttgtgct 420
cagcagtcca acagcacaga tgctagcagc agaggctcgc ctggtggtga ggctagcgga 480
gcgtgtggga ctgtaccgca agaggagcta tccccacgaa gtccagttct ttgacttgag 540
gctccttttc ctgctaacag cccttcgcac ggatgtgcgc cagcaactgt ttcaggagct 600
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aaaccctccg gtgatgcttc cagcccaaga gacggagagg gccatggaga tcctcaaagt 720
gctctttaat atcacctttg actctgtcaa gagggaagtt gatgaggaag atgctgccct 780
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ggatgtgctt ctggccctgg agctccacga aggatcctta gagttcatgg gagttaacat 960
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SEQ ID NO: 4

Length: 530

Type: PRT

Organism: Mus musculus

Sequence: 4

Met Glu Pro Arg Ala Val Ala Asp Ala Leu Glu Thr Gly Glu Glu Asp
1 5 10 15

Ala Val Thr Glu Ala Leu Arg Ser Phe Asn Arg Glu His Ser Gln Ser
20 25 30

Phe Thr Phe Asp Asp Ala Gln Gln Glu Asp Arg Lys Arg Leu Ala Lys
35 40 45

Leu Leu Val Ser Val Leu Glu Gln Gly Leu Ser Pro Lys His Arg Val
50 55 60

Thr Trp Leu Gln Thr Ile Arg Ile Leu Ser Arg Asp Arg Ser Cys Leu
65 70 75 80

Asp Ser Phe Ala Ser Arg Gln Ser Leu His Ala Leu Ala Cys Tyr Ala
85 90 95

Asp Ile Thr Val Ser Glu Glu Pro Ile Pro Gln Ser Pro Asp Met Asp
100 105 110

Val Leu Leu Glu Ser Leu Lys Cys Leu Cys Asn Leu Val Leu Ser Ser
115 120 125

Pro Thr Ala Gln Met Leu Ala Ala Glu Ala Arg Leu Val Val Arg Leu
130 135 140

Ala Glu Arg Val Gly Leu Tyr Arg Lys Arg Ser Tyr Pro His Glu Val
145 150 155 160

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Gln Phe Phe Asp Leu Arg Leu Leu Phe Leu Leu Thr Ala Leu Arg Thr
                165                 170                 175

Asp Val Arg Gln Gln Leu Phe Gln Glu Leu His Gly Val Arg Leu Leu
            180                 185                 190

Thr Asp Ala Leu Glu Leu Thr Leu Gly Val Ala Pro Lys Glu Asn Pro
        195                 200                 205

Pro Val Met Leu Pro Ala Gln Glu Thr Glu Arg Ala Met Glu Ile Leu
    210                 215                 220

Lys Val Leu Phe Asn Ile Thr Phe Asp Ser Val Lys Arg Glu Val Asp
225                 230                 235                 240

Glu Glu Asp Ala Ala Leu Tyr Arg Tyr Leu Gly Thr Leu Leu Arg His
                245                 250                 255

Cys Val Met Val Glu Ala Ala Gly Asp Arg Thr Glu Glu Phe His Gly
            260                 265                 270

His Thr Val Asn Leu Leu Gly Asn Leu Pro Leu Lys Cys Leu Asp Val
        275                 280                 285

Leu Leu Ala Leu Glu Leu His Glu Gly Ser Leu Glu Phe Met Gly Val
    290                 295                 300

Asn Met Asp Val Ile Ser Ala Leu Leu Ala Phe Leu Glu Lys Arg Leu
305                 310                 315                 320

His Gln Thr His Arg Leu Lys Glu Cys Val Ala Pro Val Leu Asn Val
                325                 330                 335

Leu Thr Glu Cys Ala Arg Met His Arg Pro Ala Arg Lys Phe Leu Lys
            340                 345                 350
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Ala Gln Val Leu Pro Pro Leu Arg Asp Val Arg Thr Arg Pro Glu Val
355 360 365

Gly Asp Leu Leu Arg Asn Lys Leu Val Arg Leu Met Thr His Leu Asp
370 375 380

Thr Asp Val Lys Arg Val Ala Ala Glu Phe Leu Phe Val Leu Cys Ser
385 390 395 400

Glu Ser Val Pro Arg Phe Ile Lys Tyr Thr Gly Tyr Gly Asn Ala Ala
405 410 415

Gly Leu Leu Ala Ala Arg Gly Leu Met Ala Gly Gly Arg Pro Glu Gly
420 425 430

Gln Tyr Ser Glu Asp Glu Asp Thr Asp Thr Glu Glu Tyr Arg Glu Ala
435 440 445

Lys Ala Ser Ile Asn Pro Val Thr Gly Arg Val Glu Glu Lys Pro Pro
450 455 460

Asn Pro Met Glu Gly Met Thr Glu Glu Gln Lys Glu His Glu Ala Met
465 470 475 480

Lys Leu Val Asn Met Phe Asp Lys Leu Ser Arg His Arg Val Ile Gln
485 490 495

Pro Met Gly Met Ser Pro Arg Gly His Leu Thr Ser Leu Gln Asp Ala
500 505 510

Met Cys Glu Thr Met Glu Gly Gln Leu Ser Ser Asp Pro Asp Ser Asp
515 520 525

Pro Asp
530

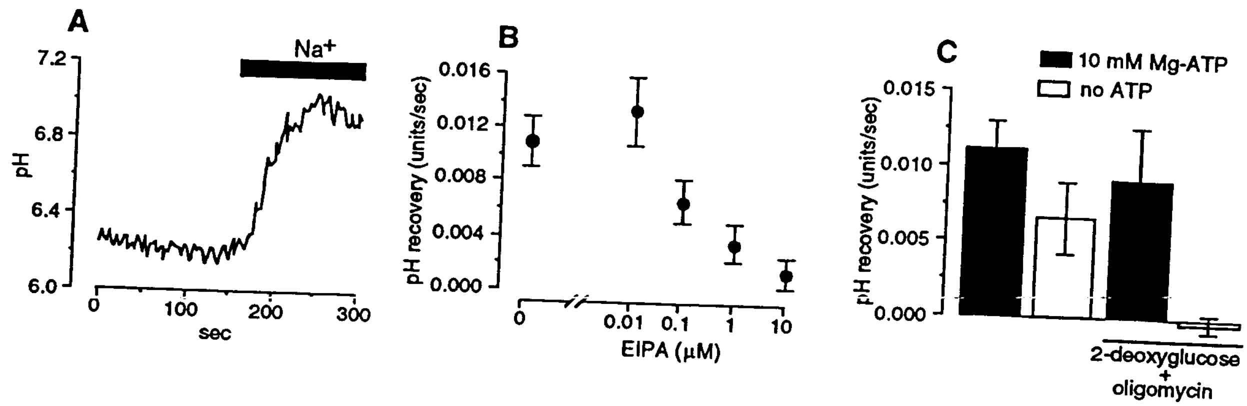
It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this sixteenth day of August 1999

THE UNIVERSITY OF SYDNEY
Patent Attorneys for the Applicant:

F B RICE & CO

Figure 1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Na+
7.2
6.8
6.4
6.0
pH
0
100
200
300
sec
B
0.016
0.012
0.008
0.004
0.000
pH recovery (units/sec)
0
0.01
0.1
1
10
EIPA (μM)
C
10 mM Mg-ATP
no ATP
0.015
0.010
0.005
0.000
pH recovery (units/sec)
2-deoxyglucose
+
oligomycin

Figure 2

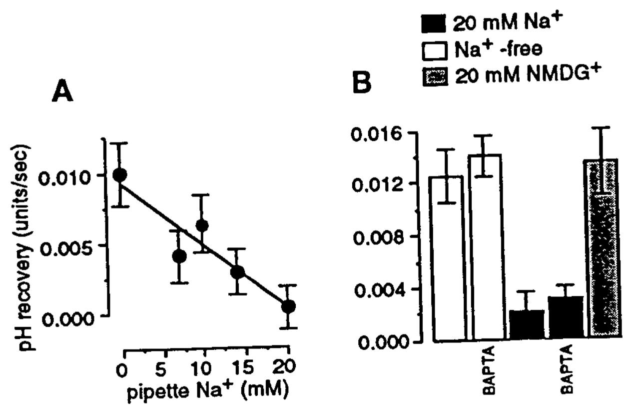

A
pH recovery (units/sec)
0.010
0.005
0.000
0 5 10 15 20
pipette Na+ (mM)
B
20 mM Na+
Na+ -free
20 mM NMDG+
0.016
0.012
0.008
0.004
0.000
BAPTA
BAPTA

Figure 3

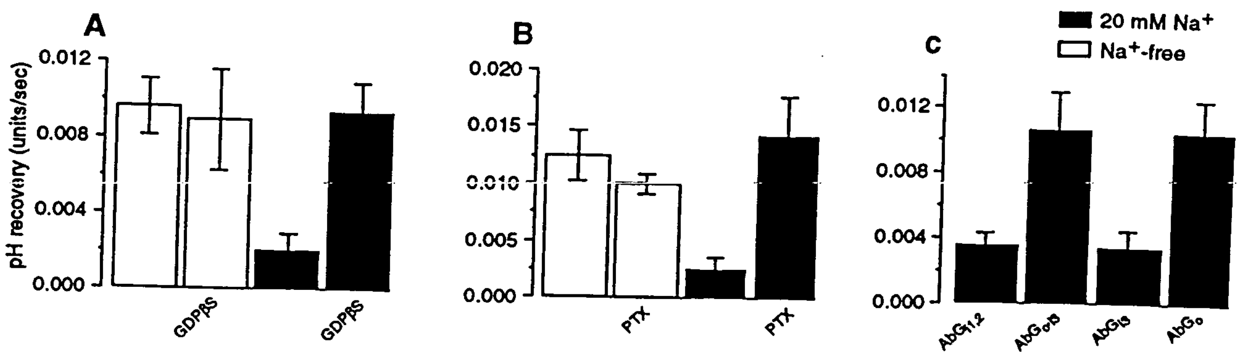
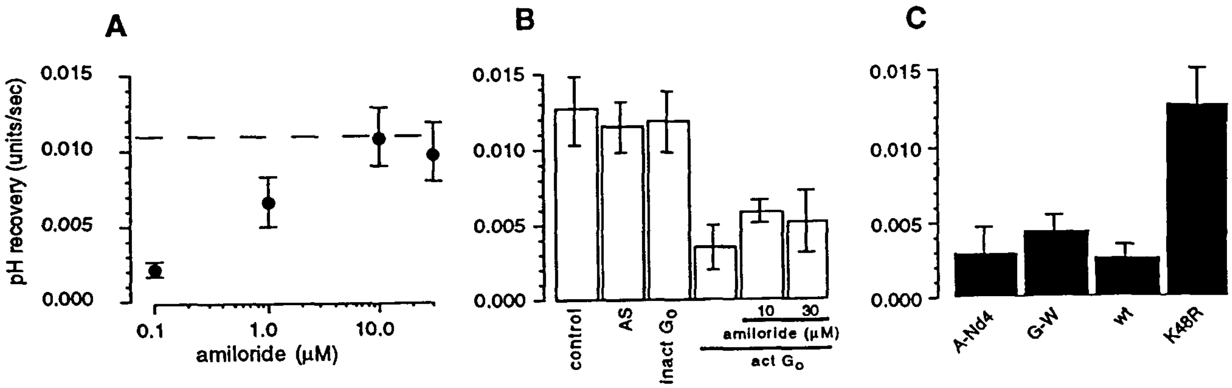

A
B
C
20 mM Na+
Na+-free
pH recovery (units/sec)
0.012
0.008
0.004
0.000
GDPβS
GDPβS
0.020
0.015
0.010
0.005
0.000
PTX
PTX
0.012
0.008
0.004
0.000

Figure 4


A
B
C
pH recovery (units/sec)
0.015
0.010
0.005
0.000
0.1
1.0
10.0
amiloride (μM)
control
AS
inact Go
10
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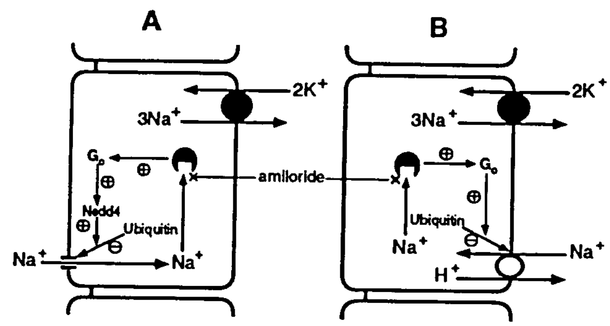
amiloride (μM)
act Go
A-Nd4
G-W
wt
K48R

Figure 5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B
2K+
3Na+
Go
Nedd4
Ubiquitin
Na+
amiloride
2K+
3Na+
Go
Ubiquitin
Na+
Na+
H+